Tuberculosis: drug resistance, fitness, and strategies for global control

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REVIEW

Tuberculosis: drug resistance, fitness, and strategies for global control

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Abstract Directly observed standardized short-course chemotherapy (DOTS) regimes are an effective treatment for drug susceptible tuberculosis disease. Surprisingly, DOTS has been reported to reduce the transmission of multi-drug resistant tuberculosis, and standardized short-course chemotherapy regimens with first-line agents have been found to be adequate treatments for some patients with drug resistant tuberculosis, including multi-drug resistance. These paradoxical observations and the apparent heterogeneity in treatment outcome of multi-drug resistant tuberculosis when using standard regimens may be due in part to limitations of in vitro drug susceptibility testing based on unique but mistakenly used techniques in diagnostic mycobacteriology. Experimental data and mathematical models indicate that the fitness cost conferred by a resistance determinant is the single most important parameter which determines the spread of drug resistance. Chromosomal alterations that result in resistance to first-line antituberculosis agents, e.g. isoniazid, rifampicin, streptomycin, may or may not be associated with a fitness cost. Based on work in experimental models and from observations in clinical drug resistant isolates a picture emerges in which, among the various resistance mutations that appear with similar rates, those associated with the least fitness cost are selected in the population.

Keywords Tuberculosis · Resistance · Treatment · Prevention · Fitness · Susceptibility testing

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Tuberculosis (TB) has been and still is one of the most common infectious causes of death on earth [16]. Treatment of tuberculosis disease faces three problems: (1) interruption of further transmission, (2) curing the acute disease, and (3) preventing relapse (most relapses occur within 6–12 months after completion of therapy). A number of landmark historical clinical studies [e.g. 9, 30, for review 32] have defined the principles which form the basis for successful drug treatment of tuberculosis disease and have resulted in the current concept of standard short-course chemotherapy (SSC). Today, the most commonly used standard chemotherapy of tuberculosis is a combination therapy consisting of a total of six months of drug treatment. Combination therapy is necessary for successful treatment of the acute infection and for prohibiting resistance to emerge, while a minimum treatment length of 6 months is required to prevent relapse of the disease. The ongoing TB pandemic is a serious threat, in particular for the developing countries, which carry most of its burden. Worldwide, the current situation is characterized by increasing numbers of drug-susceptible tuberculosis disease and by emerging drug resistance [46, 47, 50, 54]. Much attention has focused on the burden of multi-drug resistant (MDR) TB [18, 47, 55]. At present, MDR-TB continues to be a significant problem, not only in the developing countries, but also in the Baltic region, parts of the former Soviet Union, and other areas of the world.

Drug resistance and susceptibility testing in the laboratory

Plasmid-mediated mechanisms of resistance are absent in tuberculous mycobacteria, as acquired drug resistance is exclusively due to chromosomal alterations such as mutations or deletions. These chromosomal alterations affect either the drug target itself or bacterial enzymes activating the prodrug. During the past 15 years, significant knowledge has been gained concerning the mechanisms of mycobacterial drug resistance at the molecular level [for review see 36]. Isoniazid is a prodrug that requires activation by the catalase-peroxidase enzyme encoded by the *katG* gene. Resistance to isoniazid is mainly due to alterations within *katG*. In contrast, resistance to rifampicin or streptomycin is due to mutational alterations in genes encoding the drug target such as *rpoB, rpsL*, or *rrs*.

MDR-TB by definition implies resistance to at least isoniazid and rifampicin, the two cornerstone drugs of standard short-course therapy. A treatment based on isoniazid and rifampicin can not be expected to cure or substantially improve tuberculosis in patients infected with MDR-TB, nor should ineffective treatment reduce the transmission of multi-drug resistant tuberculosis. However, implementation of the DOTS program should reduce acquisition of drug resistance generated by erratic, unsupervised therapy and by an unreliable drug supply. When applied to populations with high rates of existing drug resistance, DOTS has been found to further amplify resistance [33]. In epidemiological terms, treating all patients with standard short-course therapy alone will suppress drug-susceptible strains and select for transmission of drug-resistant strains at a population level. Surprisingly, standard short-course therapy has been found to be an effective cure for 30–50% of patients with MDR-TB [19], and implementation of DOTS has been reported to reduce transmission of MDR-TB [17].

How to explain these counterintuitive observations? Meta-analyses of the impact of drug resistance on treatment outcome and transmission are complicated by the use of different methods and drug concentrations for phenotypic drug susceptibility testing in various countries. For the first-line antituberculosis drugs, there is a correlation between the drug susceptibility testing result in-vitro and the clinical usefulness of the drug. Based on historical data gathered in the 1960s [11, 12], there has been a generally accepted consensus on how laboratory testing of drug susceptibility of *Mycobacterium tuberculosis* should be performed [23, 42]. The definition of resistance in the mycobacteriology laboratory dates back to 1962: "Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug". This definition is an epidemiological one. Already in 1969 the prognostic significance of in vitro determined drug resistance had been found to be limited. "There is evidence that the presence of resistance to a single drug has little or no effect on the outcome of treatment with the three drugs isoniazid, streptomycin, and para-amino salicylic acid. Furthermore, even in the presence of primary resistance to two first-line drugs, a bacteriological response is not infrequently obtained with the three drug regimen" [11].

In the diagnostic laboratory, a single drug concentration, termed "critical concentration", is primarily used for drug susceptibility testing and to categorize a clinical isolate of *M. tuberculosis* as susceptible or resistant [23]. This "critical concentration", however, bears little relationship to the drug concentrations which are present in vivo in the patient (see Table 1), e.g. the serum concentrations for isoniazid and streptomycin are 10- to 20-fold higher compared to the "critical concentration". This contrasts with common procedures established in antibiotic therapy of infectious diseases which take pharmacokinetic properties into account and where the relationship between phenotypic resistance in vitro and drug concentration in vivo is addressed by the definition of breakpoints. Thus, the resistance phenotype determined in vitro is related to the drug levels which are present in vivo. What is needed in diagnostic mycobacteriology are standardized measures of quantitative drug susceptibility testing. For example, isolates categorized as resistant according to the "critical concentration", should be subjected to determination of minimal inhibitory concentrations.

For streptomycin and isoniazid, a significant fraction of clinical TB isolates categorized as resistant in the diagnostic laboratory exhibits only a low-level resistant phenotype [31, 51]. A successful treatment outcome despite a resistant phenotype—as determined by routine drug susceptibility testing—most likely reflects limitations of the procedures used to determine drug susceptibility and indicates that

Table 1 Mycobacterial drug susceptibility testing—the critical concentration

Antimicrobial agent	MIC [mg/l] of susceptible *M. tuberculosis*	Concentration [mg/l] in serum	Concentration [mg/l] used for testing	
			Low	High
Isoniazid	0.05–0.2	7	0.1	0.4
Rifampicin	0.5	10	2	–
Pyrazinamid	20	45	100	–
Ethambutol	1–5	2–5	2.5	7.5
Streptomycin	1	25–50	2	6

low-level drug resistance may not correspond to clinical resistance [6, 15, 37]. The NCCLS subcommittee has incorporated parts of these considerations in its guidelines. "In the case of isoniazid, if an isolate is resistant to the critical concentration of 0.1 μg/ml but susceptible to the higher concentration of 0.4 μg/ml, the following comment should be given—the test results indicate low-level resistance to isoniazid: some evidence suggests that patients infected with corresponding strains may benefit from continuing therapy with isoniazid" [42]. According to results from systematic quantitative drug susceptibility testings at the National Center for Mycobacteria (IMM, University of Zurich), more than one-third of clinical TB strains categorized as resistant to isoniazid in Switzerland exhibit a low-level resistant phenotype with MIC values less than 1.0 mg/l. In contrast, rifampicin resistance, predominantly corresponds to a high-level drug resistant phenotype with MIC values >50 mg/l (our own unpublished data). These data lead to the hypothesis that some strains categorized by in vitro drug susceptibility testing as MDR-TB strains may not correspond to clinical multi-drug resistance, in vivo. Treatment of corresponding infections with a standard short-course regimen is likely to cure the acute disease, thus providing an adequate explanation for the seemingly paradoxical observations that DOTS represents not only an effective treatment for a significant fraction of MDR-TB disease, but also reduces transmission of primary MDR-TB. It remains unclear, however, whether SSC based regimens will effectively prohibit relapse under these conditions.

The consequences of erratic drug susceptibility testing are particular severe in terms of treatment options for apparent MDR or XDR tuberculosis. Isoniazid resistance reported by the diagnostic laboratory may lead to the use of second or even third-line antibiotics—compounds which are compromised by severe toxicity and which almost certainly have inferior activity than isoniazid against low-level INH resistant strains. The thioamide drugs, ethionamide (ETH) and prothionamide (PTH), are reasonable treatment options for MDR tuberculosis. Isoniazid and the thioamide drugs share InhA as the primary target of action [36, 52]. In contrast to isoniazid, the thioamides do not require activation by KatG. Thus, strains with high-level isoniazid resistance due to mutational KatG alterations typically retain thioamide susceptibility. Newly introduced molecular diagnostic tests (e.g. the GenoType MTBDR assay) offer rapid determination of genotypic resistance, as they allow for the direct detection of the most frequent and relevant *rpoB*, *katG* and *inhA* resistance mutations in smear-positive specimens [40]. These tests may assist in therapeutic decisions in treatment of drug-resistant tuberculosis, e.g. whether to use isoniazid or the alternative thioamide drugs.

Compared to isoniazid, rifampicin, and streptomycin, drug susceptibility testing for ethambutol is particularly problematic [28]. A number of reasons may account for this, e.g. the bacteriostatic nature of ethambutol and reduced activity of the drug in culture medium. Most important, however, is the small difference between the drug concentration used for in vitro drug susceptibility testing and the natural drug susceptibility of wild-type isolates of *M. tuberculosis* (see Table 1). Thus, minute changes in drug susceptibility will have a major impact on the interpretation of the in vitro test result, with only a narrow range between MICs of susceptible and MICs of resistant isolates of *M. tuberculosis*. Despite identification of the *emb* gene cluster, proposed to encode for a mycobacterial arabinosyl transferase, as a target for ethambutol [2], the role of the *emb* operon in resistance is unclear [43]. In particular, the association of *emb*B codon 306 mutation with ethambutol resistance in *M. tuberculosis* remains enigmatic [21, 24, 34]. This is not the least due to the lack of clear-cut gene replacement experiments in *M. tuberculosis* using the codon 306 mutant *embB* allele.

Drug resistance and fitness

A common perception in drug resistance implies that drug resistance has a cost: a drug resistance determinant provides an advantage in the presence of the drug, but in the absence of the drug the resistance determinant is associated with a fitness burden [5, for review 1]. In mathematical models, the fitness cost of drug resistance is the primary parameter that determines both the frequency of resistance at any given level of antibiotic use and the rate at which that frequency will change with changes in antibiotic use patterns. Experimentally, in the laboratory, it has been demonstrated that compensatory mutations may occur which counteract the fitness burden associated with a primary resistance determinant. These compensatory mutations have been suggested to maintain the spread of resistance even in the absence of antibiotics [1]. In case a fitness lowering chromosomal alteration occurs, two possibilities thus exist: (1) the mutant carrying the chromosomal alteration becomes extinct, or (2) the chromosomal alteration is fixed in the population by means of a compensatory evolution.

A different and particularly instructive picture emerged when studying the mechanisms of streptomycin resistance in *M. tuberculosis* [7]. The results of these studies question the dogma of a resistance-associated fitness cost. These investigations were the first to combine data on the frequency of molecular resistance determinants in clinical isolates in vivo with in vitro experimental data on the genetics and costs associated with a resistance determinant.

The procedure involved three steps: (1) to determine the frequency of resistance mutations in drug resistant *M. tuberculosis* strains isolated from patients versus the frequency in in vitro selected drug resistant mutants, (2) to introduce resistance determinants by means of genetic techniques into a suitable model to obtain isogenic mutants, and (3) to experimentally determine the fitness cost of a resistant determinant in an in vitro competition growth assay. In vitro, a variety of mutations in either ribosomal protein S12 or the small subunit rRNA result in resistance to streptomycin. However, in vivo a strict correlation was found between the frequency of a given resistance mutation in clinical isolates and its fitness cost as determined in vitro. The no-cost Lys → Arg alteration at position 42 of *rpsL* is by far the most frequent streptomycin resistance mutation in clinical isolates (see Table 2). An important control was to determine the stochastic probability of the different resistance mutation in vitro: no such correlation is found here [8, 38]. Thus, while the stochastic probabilities of the different resistance mutations may be similar, there is a significant selection in vivo for those resistance mutations which carry no fitness cost.

Crystallographic data on ribosome-drug complexes have helped to understand the fitness cost associated with the various streptomycin resistance mutations [13]. Most of the streptomycin resistance mutations will affect the fidelity of translation by leading to ribosomal hyper-accuracy. Among the different *rpsL* mutations conferring resistance to streptomycin, e.g. Lys 42→Arg, Thr or Asn, the replacement of lysine by arginine at aa position 42 is the only mutation known that confers resistance to streptomycin without affecting the fidelity of translation [26]. Mutation of lysine to arginine would disrupt hydrogen-bonding contacts to the OH groups of streptomycin (see Fig. 1) and thereby reduce the affinity of the ribosome for streptomycin, leading to resistance. Compared to the streptomycin resistance mutations RpsL aa 42 Lys→Thr or Asn, however, the Lys→Arg alteration does not affect

Table 2 Frequency of genotypic alterations in clinical *M. tuberculosis* isolates resistant to streptomycin[a]

Mutations	Frequency in clinical isolates	Mean relative fitness	MIC mg/L
rpsL 42 Arg	88%	0.98	>1,000
rrs 523 C	6%	0.94	125
rrs 522 T	3%	0.91	250
Rrs 526 T	2%	0.90	125
rpsL 42 Thr	<1%	0.86	>1,000
rpsL 42 Asn	<1%	0.85	>1,000

[a] Resistance mutations in *rpsL* aa position 88 and in *rrs* positions 501, 912, and 913 are not included; these mutations account for approximately 10% of clinical streptomycin resistant isolates

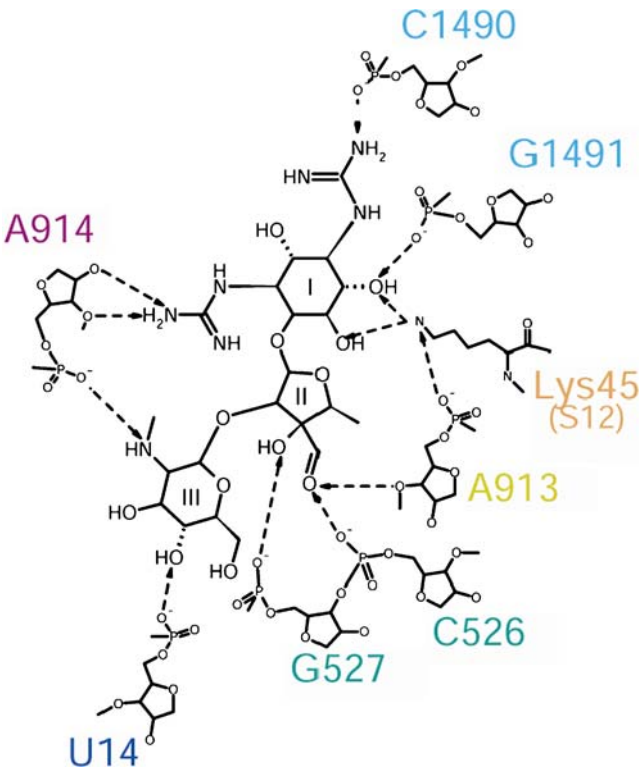

Fig. 1 Chemical structure of streptomycin, showing interaction of the various groups with specific residues of the *Thermus thermophilus* small ribosomal subunit; nucleotides correspond to 16S rRNA, Lys 45 (S12) corresponds to ribosomal protein *RpsL* aa position 42. Figure taken from Ref. 13 with permission of the publisher

intra-ribosomal contacts of aa 42 to small subunit RNA nucleotides and thus translation remains normal.

These recent experimental results addressing the molecular mechanisms of resistance and fitness cost are corroborated by epidemiological observations from some 40 years ago [10]. In his investigations, Canetti studied primary versus acquired drug resistance in *M. tuberculosis*. Primary resistance is defined as infection with a resistant strain, here a resistant strain is transmitted from patient to patient. Acquired resistance is defined as resistance developing in a patient following infection with a drug susceptible strain. "Primary resistance is not a mere replication of acquired resistance. Other factors, such as altered virulence of the resistant strains, or instability inherent to certain types of resistance, may also be at work in producing the difference. If some of the strains with acquired resistance are incapable (through insufficient virulence) of producing new cases of tuberculosis .... the relative frequency of resistance must necessarily be lower in primary than in acquired resistance" [10].

Canetti categorized streptomycin resistance into low-level (≥4 μg/ml), intermediate-level (≥10 μg/ml) and high-level (≥100 μg/ml) drug resistance (see Table 3). At this gross level, there was no significant difference between

Table 3 Primary resistance and acquired resistance to streptomycin

	Total cases		Concentration of streptomycin [μg/ml]					
			≥4		≥10		≥100	
	n	%	*n*	%	*n*	%	*n*	%
Primary resistance	163	100	94	58	34	21	35	21
Acquired resistance	426	100	267	63	102	24	57	12

primary and acquired drug resistance with respect to the relative proportions of the different streptomycin resistance levels. In other words, within streptomycin resistant mutants high-level drug resistance mutations must exist which did not impede transmission. For rifampicin, the situation is similar. Different mutations in *rpoB* result in drug resistance [4, 20, 22, 29]. As with streptomycin, a significant correlation exists between the frequency of a particular mutation in clinical isolates and the fitness cost associated with the mutation (see Table 4).

For isoniazid, the situation is different. Compared to acquired resistance, the high-level resistance phenotype is significantly underrepresented in primary resistance (11% versus 53%, see Table 5). Apparently, high-level isoniazid resistance is associated with a significant fitness burden which impedes transmission. Multiple chromosomal alterations in *katG* may result in resistance to isoniazid. It was demonstrated some 50 years ago that high-level resistance to isoniazid—as conferred by deletion of *katG*, i.e. complete loss of katalase-peroxidase activity—is associated with a significant fitness cost [14]. More recently, it was shown that complete loss of KatG activity in clinical isoniazid resistant strains is associated with a secondary mutation resulting in over-expression of the alkyl-hydroperoxide-reductase AhpC [39]. It was hypothesized that in strains with isoniazid resistance due to deletion of *katG*, compensatory mutations in *ahpC* will develop over time, ultimately facilitating transmission and spread of resistant microorganisms. The most frequent isoniazid resistance mutation found in clinical strains, however, is not a nonsense mutation, but a serine to threonine replacement at aa position 315 of KatG [48, 49]. This particular mutation confers an intermediate level of resistance and is not associated with a fitness cost [35]. The 315 Ser→Thr mutation is found in approximately 60% of clinical strains with isoniazid resistance. In the absence of KatG/isoniazid crystal complexes, modelling and computational studies have been used to understand drug–target interaction [3]. These and other studies suggest [25] that a 315 Ser→ Thr mutation in the KatG catalase–peroxidase would alter the binding site for isoniazid but retain the active site properties for proper catalytic function.

Available evidence suggests that within a spectrum of possible mutational resistance alterations each being associated with a distinct fitness cost, a selection for those resistance mutations with the least resistance-associated cost seems to exist in vivo [4, 8, 29, 35, 38, 49]. This selection is best explained by fluctuating environments, i.e. expansion of mutants experiencing a low fitness cost in the absence of antibiotics during periods in which selection for antibiotic resistance is removed. The rare finding of high cost resistance mutations in clinical isolates can be explained by the stochastic probability of a resistance mutation in a size limited bacterial population and by bottleneck phenomena which take place in transmission. It is under such conditions that compensatory mutations which ameliorate the cost of resistance are likely to occur [38].

A priori, there is no need for compensatory evolution in maintaining persistence and further spread of drug resistance, as resistance mutations exist which carry little or no fitness cost at all. As long as for a given drug different resistance mutations exist with only one of these being a no-cost resistance mutation, the stochastic probability of selection for this particular mutation in a given population is much higher than the probability of two mutations occurring either simultaneously or successively with one compensating for disadvantages conferred by the other. The frequent presence of and selection for drug resistance mutations which carry no or only a low fitness cost, also indicates that drug persistence per se can not be expected to restrict transmission of tuberculosis disease.

Table 4 Frequency of *rpoB* mutations in clinical *M. tuberculosis* isolates resistant to rifampicin[a]

RpoB mutation	Frequency in clinical isolates	Mean relative fitness	MIC mg/l
S 531 L	54%	0.93	>32
H 526 Y	11%	0.82	>32
H 526 D	7%	0.78	>32
S 531 W	4%	0.79	>32
H 526 R	3%	0.56	>32
S 522 L	1%	0.54	16–32

[a] Data tabulated from [4, 20, 22, 29]

Table 5 Primary resistance and acquired resistance to isoniazid

	Total cases		Concentration of isoniazid [μg/ml]					
			≥0.2		≥1		≥10	
	n	%	*n*	%	*n*	%	*n*	%
Primary resistance	98	100	49	50	38	39	11	11
Acquired resistance	443	100	114	26	95	21	234	53

Limitations of the DOTS strategy

In 1994, the WHO declared tuberculosis a global emergency and introduced the DOTS strategy for global tuberculosis control. However, the plan has produced variable success and despite intensified efforts to diagnose and treat tuberculosis, the rates continue to climb in some regions. Although various experts have argued that the failure of the DOTS strategy to control tuberculosis resulted from failed implementation, poor public health infrastructure, poverty, and the like, recent reports indicate that the problem with the DOTS strategy may be a more principal one [45, 53]. For example, tuberculosis rates were found to increase despite implementation of DOTS [27]. The DOTS strategy is built around five activities: (1) case detection by sputum smear microscopy among symptomatic patients self-reporting to health services, (2) directly observed therapy using standard short-course regimens, (3) regular supply of medication, (4) governmental commitment to sustained tuberculosis control by providing resources and infrastructural capacity, and (5) a standardized recording and reporting system that allows assessment of individual treatment results and of the tuberculosis control program overall.

The reproductive rate of an epidemic is characterized by three determinants: (1) the duration of infectiousness of an infected patient, (2) the number of contacts between an infectious patient and susceptible contacts, and (3) the probability of the infected to become infectious. As a treatment program, DOTS in part targets the duration of infectiousness. However, DOTS does not address the number of contacts between an infectious patient and susceptible contacts before diagnosis on the basis of passive case-finding [44], nor does it address the probability of the infected to become infectious. It has been estimated that approximately one tenth of those infected develop active disease and half of those with active disease become themselves infectious [41]. Thus, it would be necessary for one tuberculosis case to infect approximately twenty susceptible contacts in order to produce another infectious case and to maintain the same level of infection in the population. Any measure which decreases this number will result in a decline of tuberculosis rates. Two simple calculations may serve to illustrate the limitations of a mere treatment program based on passive case finding. First, a patient, prior to detection on the basis of passive case finding, infects more than 20 susceptible contacts before transmission is interrupted by treatment. This is a scenario which is quite conceivable under the living conditions present in many of the world's underdeveloped areas. Second, rather than 10%, a significantly larger fraction of the infected develops active disease, due to either inherent genetic (disease susceptibility and genetic polymorphisms), societal (poverty and starvation), or environmental reasons, e.g. coinfection with HIV. The latter is particularly worrisome due to the widespread nature of the AIDS epidemic and its association with a high risk of developing progressive primary tuberculosis. Under these circumstances, the DOTS program will have significant shortcomings in the global control of TB.

More data are needed to estimate our case finding ability in various settings. Available data indicate that in high burden environments it may be quite low, e.g. in South Africa. In that case, no strategy will reduce incidence, unless case finding ability is enhanced [45]. The current WHO recommendations form an excellent base for tuberculosis control, but may need to be complemented by a strategy which reflects the biology of the disease by considering determinants which influence the reproductive rate of an epidemic. The DOTS program as a treatment program is ideally combined with a component which actively targets transmission, designed to interrupt the spread of *M. tuberculosis* in the community. Such a component could be the implementation of contact tracing and active case finding. Can we afford to do it, or rather, can we afford to ignore it?

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